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Replication of Mengovirus in HeLa Cells Preinfected with Nonreplicating Poliovirus

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The replication of mengovirus in HeLa cells preinfected with poliovirus in the presence of 10^{-3} M guanidine was investigated. Although host cell protein synthesis is inhibited by the presence of nonreplicating poliovirus, it is found that mengovirus ribonucleic acid (RNA) and protein synthesis proceed normally under the same conditions. Furthermore, no effects on mengovirus growth by poliovirus can be detected either when Mengo protein synthesis is interrupted by Acti-Dione or when its RNA synthesis is reduced by incubation at 28 C. It is suggested that the poliovirus inhibitory factor may be able to distinguish between an RNA element required in the protein-synthesizing apparatus of the host cell and a comparable element in that of the heterologous virus.

Recent experimental observations indicate that infection of cells by picornavirus is accompanied by a selective inhibition of the host cell protein synthesis. The inhibition appears to be mediated by a protein produced by the virus subsequent to entry into the cell (6). While host-directed protein synthesis is decreasing, viral specific protein synthesis is increasing (5). Apparently the viral-directed protein synthesis is insensitive to the factor which inhibits host cell synthesis.

We have been investigating the basis of this selective inhibition and have attempted to ascertain experimentally whether it is real. It is possible that virus-directed protein synthesis is also sensitive to the virus-produced inhibitory factor, but that the rapid rate of virus-directed synthesis masks this sensitivity. In the studies reported here, conditions of viral infection are chosen which make it possible to determine whether the viral protein-synthesizing apparatus is refractory to viral-induced protein synthesis inhibition.

The best system for investigating the possible sensitivity of virus-directed protein synthesis to virus-induced inhibition appeared to be superinfection of poliovirus-infected cells with a guanidine-resistant virus. In this situation, poliovirus can be prevented from replicating by the use of guanidine, but it is capable of causing a rapid inhibition of host cell-specific protein synthesis (7). The effect of this inhibition on the subsequent growth of the heterologous superinfecting virus can be studied in detail.

Cords and Holland (2) have already shown that superinfection of cells, preinfected with polio in the presence of guanidine, with a heterologous guanidine-resistant virus leads to relatively normal growth of the resistant virus. These authors use the production of infectious ribonucleic acid (RNA) as an indicator of the growth of the heterologous virus. Choppin and Holmes (1) have also shown that SV5 RNA replicates in polio-infected cells.

In the experiments presented here, the incorporation of uridine into viral-specific RNA is used to measure precisely the kinetics of the growth of mengovirus in preinfected and uninfected control cells. In addition, conditions of incubation are used which inhibit either viral protein synthesis or viral-specific RNA synthesis. In these latter experiments, the mengovirus protein-synthesizing system is exposed to the inhibitory factor of polio for long periods of time without being able to replace either protein or RNA components. Thus, any sensitivity of mengovirus to inhibition by polio is not masked by rapid synthesis by mengovirus, and it can be concluded that the superinfecting virus is, indeed, absolutely insensitive to any inhibitory function expressed by poliovirus.

MATERIALS AND METHODS

HeLa S-3 cells were grown in suspension in Eagle's medium with 7% horse serum (3). The preparation of the poliovirus and mengovirus stocks used in these experiments has been described before (4). Unless otherwise stated, the medium used in all experiments contained 10^{-3} M guanidine hydrochloride, a drug which specifically inhibits poliovirus replication without affecting mengovirus development.

To observe the multiplication of mengovirus in HeLa cells preinfected with poliovirus, 25 ml of cells growing at 4×10^5 cells per ml were concentrated to 1.2×10^7 cells per ml in Eagle's medium with 10^{-3} M guanidine and infected with poliovirus at a multiplicity of about 100 plaque-forming units (PFU) per cell. A control culture received an equal volume of medium. After an attachment period of 30 min at 37 C, the cells were centrifuged for 2 min at $800 \times g$, and the cell pellet was resuspended in Eagle's medium supplemented with 10% horse serum and 10^{-3} M guanidine to give a concentration of 4.4×10^6 cells per ml. After 2.0 hr of additional incubation at 37 C, the cells were infected with mengovirus at a multiplicity of about 20 PFU per cell. At this time, actinomycin was also added to a concentration of 5 μg/ml; 30 min later, 0.25 μc of ^{14}C-uridine, with a specific activity of 25.5 mc/mmole (Schwarz Bio Research Inc., Orangeburg, N.Y.), was added to each 2.5 ml of culture. Samples (0.1 ml) were withdrawn from the cultures at regular intervals and prepared for radioactive assay as described previously (4).

The determination of mengovirus-specific protein synthesis in cells preinfected with poliovirus was made in the following manner. The procedure for infections was as described above. At various times, samples containing 8×10^5 cells were withdrawn from each culture and placed in 4.0 ml of ice-cold Earle's solution. This suspension was centrifuged at 4 C at $800 \times g$ for 2 min in an International PR-2. The cell pellets were warmed to 37 C and then rapidly resuspended in 0.5 ml of Eagle's medium (37 C) containing one-fifth the normal leucine concentration, 10% horse serum, and 0.8 μc of ^{14}C-leucine (specific activity, 200 mc/mmole) per ml (Schwarz Bio Research, Inc.) The suspensions were incubated for 5 min at 37 C. At this time, a 0.3-ml sample was withdrawn and added to 4.0 ml of ice-cold Earle's solution to terminate incorporation. The samples were centrifuged at 4 C for 5 min at $800 \times g$ and the cell pellets were resuspended in 0.5 ml of 1 N KOH. After 10 min of incubation at room temperature, the samples were precipitated with 1.0 ml of 20% trichloroacetic acid. The precipitates were collected on filters (Millipore Corp., Bedford, Mass.) after four washings with 5% trichloroacetic acid and counted in a Picker Magnachanger gas-flow counter.

Assays of poliovirus-induced inhibition of HeLa cell protein synthesis at 37 and 28 C were performed by measuring the rate of ^{14}C-leucine incorporation according to the method described above, except that the ^{14}C-leucine concentration was 0.4 μc/ml instead of 0.8 μc/ml.

Actinomycin D was a gift of Merck Sharp and Dohme (Rahway, N.J.), and Acti-Dione was purchased from Calbiochem (Los Angeles, Calif.).

RESULTS

Actinomycin-resistant RNA synthesis is a convenient index of picornavirus replication. The incorporation of ^{14}C-uridine during mengovirus development was measured to determine whether viral replication occurred in cells preinfected with poliovirus (Fig. 1). Nonreplicating poliovirus was permitted to produce its inhibitory effects for 2 hr before mengovirus infection was initiated. In cells infected with poliovirus alone, no significant incorporation occurred, because actinomycin prevents host cell RNA synthesis and the added guanidine prevents poliovirus RNA replication. It is apparent that the kinetics and rate of mengovirus RNA synthesis in both the preinfected and previously uninfected cultures are essentially identical. Although the total amount of ^{14}C-uridine incorporated at 6.5 hr was slightly higher in the control, it should be noted that this varied considerably with experiments. The important observation is that the time of mengovirus-specific RNA synthesis in preinfected cells was the same as in the control and appeared to proceed normally. The inhibitory effects of nonreplicating poliovirus known to be exerted on HeLa cell metabolism have not significantly affected mengovirus development.

Because viral RNA synthesis depends on the production of viral-specific proteins early in infection, the above result implies that mengovirus

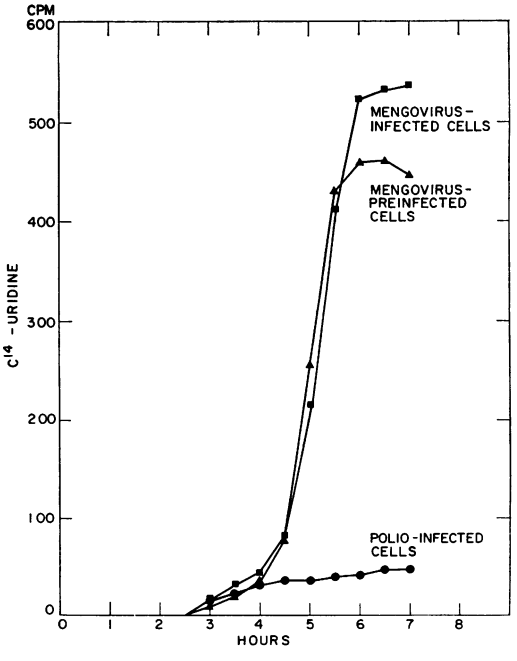

FIG. 1. *Mengovirus-specific RNA synthesis in HeLa cells preinfected with nonreplicating poliovirus. Infection with poliovirus at time zero and superinfection with mengovirus at 2 hr in the presence of 10^{-3} M guanidine and 5 μg of actinomycin per ml were carried out as described in Materials and Methods. At 2.5 hr, 0.1 μc of ^{14}C-uridine (specific activity, 25.5 μc/μmole) per ml was added.*

protein synthesis proceeded normally in cells which had previously had host protein synthesis inhibited by poliovirus. To demonstrate the concurrent inhibition of host cell protein synthesis by poliovirus and the apparent insensitivity of mengovirus protein synthesis, a pulse-labeling experiment was carried out to examine the rate of ^{14}C-leucine incorporation as a function of time in superinfected HeLa cells (Fig. 2).

In this experiment, uninfected cells showed a relatively constant rate of protein synthesis until actinomycin was added at 2 hr. Subsequent to this, there was a gradual decline in synthetic rate due to the cessation of host cell RNA production. Cells infected with poliovirus alone at zero time displayed a decline in protein synthetic rate which increased with the addition of actinomycin at 2 hr. A marked increase in protein synthesis at 4 hr occurred in the culture infected at zero-time with poliovirus and superinfected with mengovirus at 2 hr. This increase may be attributed to the production of mengovirus-specific proteins, since both poliovirus and HeLa cell protein synthesis were prevented by the conditions of this culture. Thus, mengovirus grew normally in a cell in which prior virus infection had completely inhibited cellular protein synthesis. Furthermore, plaque assay determinations (4) made on yields of mengovirus grown in superinfected and control cultures showed that viral PFU production was essentially equivalent under both conditions.

The experimental results implied that the component of the host cell protein synthesis machinery, which is inhibited by virus infection, is not a component for virus-directed protein synthesis. There is, however, another possible explanation of the results which must be ruled out. The superinfecting virus-directed protein synthesis may, in fact, involve a component which is sensitive to inhibition by the preinfecting poliovirus, but this component is replaced rapidly by the multiplying virus. Thus, the superinfecting virus would appear insensitive to inhibition by polio when, in fact, its sensitivity was masked by large rates of viral-directed synthesis.

Two types of experiments were performed to determine whether the apparent resistance of mengovirus to inhibition by poliovirus was due to the rapid production of some protein-synthesizing component by the mengovirus. The experimental design was to inhibit selectively either protein synthesis or RNA synthesis by mengovirus in cells previously infected with poliovirus. In the first experiment, protein synthesis by mengovirus was inhibited with Acti-Dione. It has been shown that, when cells are infected with poliovirus and the inhibitory effects of the virus are permitted to develop, these inhibitory effects continue to be

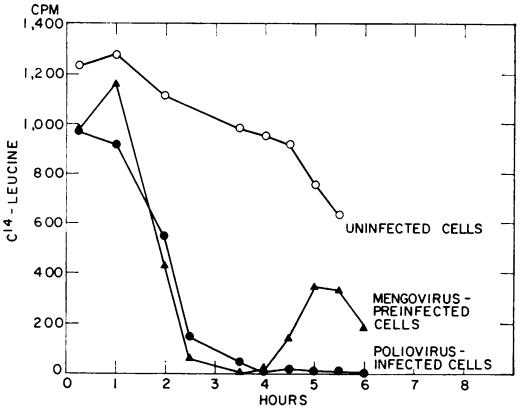

FIG. 2. *Protein synthesis in cells preinfected with poliovirus at time zero and superinfected with mengovirus at 2 hr. The rates of protein synthesis in uninfected, poliovirus-infected, and mengovirus-superinfected HeLa cells were measured at the times shown. Each culture was treated with 5 μg of actinomycin per ml at 2 hr. Infection and pulse-labeling for 5 min with ^{14}C-leucine were performed as described in Materials and Methods.*

manifested upon subsequent addition of Acti-Dione (8). Thus, once the inhibitory factor is produced by poliovirus, it continues to operate in the presence of Acti-Dione. However, the effects of the inhibition are apparent only upon subsequent removal of the drug.

In the experiment shown in Fig. 3, cells were preinfected with poliovirus, superinfected with mengovirus, and, after a period of initial mengovirus development, Acti-Dione was added and the cells were incubated. Upon removal of the drug, mengovirus grew normally except for a time lag corresponding to the length of time that Acti-Dione was present. Comparison with a control culture which received the drug but which had not been preinfected with poliovirus shows that there was no appreciable effect on the time and initial rate of mengovirus growth due to the poliovirus during the time that Acti-Dione was present. The yield of mengovirus was slightly lower in the preinfected cells than in the control culture; this was ascribed to progressive cell damage due to the presence of the preinfecting but nonreplicating poliovirus. The significant observation is that the growth of mengovirus occurred at the same time as in the control culture and with the same initial slope of Mengo-specific RNA synthesis.

The result of the experiment shown in Fig. 3 was interpreted as follows. During the period of incubation with Acti-Dione present, mengovirus can produce no proteins; therefore, any protein involved in Mengo-specific protein synthesis

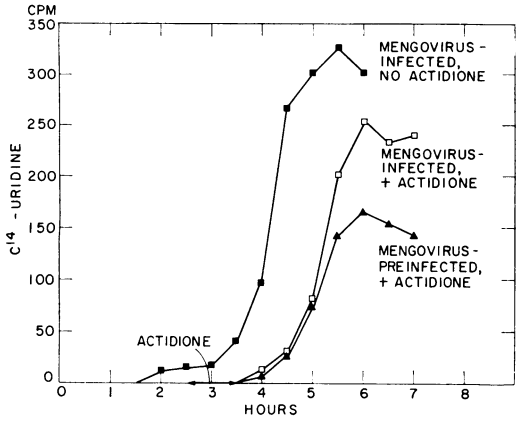

FIG. 3. *Mengovirus RNA synthesis in preinfected cells after 1 hr of incubation with 100 μg of Acti-Dione per ml. At time zero, one of three cultures was preinfected with 100 PFU of poliovirus per cell in the presence of 10^{-3} M guanidine. At 1 hr, all three cultures were infected with 20 PFU of mengovirus per cell and treated with 5 μg of actinomycin per ml. To the nonpreinfected control culture, 0.1 μc of ^{14}C-uridine (specific activity, 44 μc/μmole) per ml was added at 1.5 hr; the other two cultures were treated with 100 μg of Acti-Dione per ml between 2.5 and 3.5 hr. Upon reversal of Acti-Dione at 3.5 hr ^{14}C-uridine was added to these two cultures and RNA synthesis was assayed as for Fig. 1.*

which is sensitive to inhibition by poliovirus cannot be replaced. It would be expected, therefore, that upon removal of the drug, mengovirus growth would be retarded when compared to a control which was not preinfected. Since no appreciable effect on mengovirus growth was observed, it seems likely that there is no protein produced by mengovirus and used in protein synthesis which can be affected by poliovirus.

There remains the possibility that a species of RNA may be the target for inhibition by poliovirus. This was suggested by the observation that the addition of actinomycin to cells greatly enhances the rate at which poliovirus inhibits host-directed protein synthesis. Thus, when infected HeLa cells are permitted to replace RNA by new synthesis, host-directed protein synthesis continues for a much longer time. The replaced RNA could be messenger RNA, although recent experiments in our laboratory have indicated that it might be another species of RNA, possibly transfer RNA. To establish that mengovirus protein synthesis is insensitive to poliovirus inhibition, it was considered important to show that poliovirus had no effect on mengovirus growth even when mengovirus RNA synthesis was greatly reduced. Thus, the possibility that mengovirus produced an RNA at a higher rate than can be inactivated by poliovirus can be ruled out.

To test this hypothesis, experimental conditions were devised in which the rate of mengovirus RNA synthesis was reduced significantly, but the poliovirus-induced inhibitory activity was not greatly altered. Such a situation is obtained by reducing the culture temperatures to 28 C. The inhibition of protein synthesis by nonreplicating poliovirus at 28 C was determined by comparing the rates of ^{14}C-leucine incorporation by infected HeLa cells at 28 C and the normal culture temperature of 37 C (Fig. 4). Poliovirus infection was carried out as described above. Uninfected and infected cultures were incubated at 37 C for 75 min, at which time actinomycin was added, and the cultures were divided. Half of each culture remained at 37 C and half was shifted to 28 C. At the times shown, samples were obtained for pulse-labeling at 37 C, as described for Fig. 2. The results indicate that protein synthesis in uninfected cells was essentially unaffected by incubation at 28 C. Examination of the infected cultures revealed that the rate of viral-induced protein synthesis inhibition is only slightly reduced at 28 C as compared to the culture at 37 C. This reduction may be attributed to the decreased production of the inhibition factor as well as a decrease in the rate of the reaction of the factor with HeLa protein-synthesizing apparatus. Although there is a small decrease in the rate of viral-induced inhibition at 28 C, it is clear that incubation at this temperature allows the marked inhibition of cellular protein synthesis similar to that which occurs at 37 C.

The effect of reduced incubation temperature on mengovirus RNA synthesis is shown in Fig. 5. Four replicate cultures of HeLa cells at 4.0×10^6

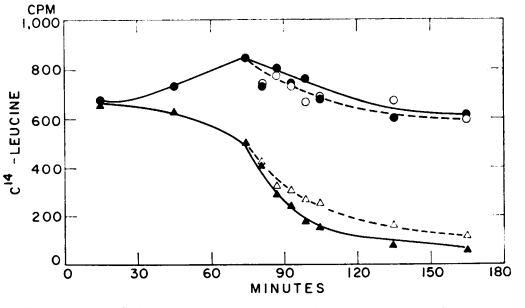

FIG. 4. *Inhibition of HeLa cell protein synthesis at 37 C and 28 C by poliovirus in the presence of 10^{-3} M guanidine. Cells were infected with poliovirus at 100 PFU/cell at time zero; the rate of protein synthesis was determined in infected and uninfected cultures. At 75 min, both cultures were treated with 5 μg of actinomycin per ml and half of each culture was shifted to 28 C. Symbols: (●) uninfected, 37 C; (○) uninfected, 28 C; (▲) infected, 37 C; (△) infected, 28 C.*

cells/ml were infected at time zero with 20 PFU/cell of mengovirus; at this time, 5 μg of actinomycin per ml was added to each culture After 30 min, ^{14}C-uridine was added; subsequently, 0.1-ml samples were withdrawn at regular intervals for radioactive assay. While one culture remained at 37 C throughout, other cultures were shifted to 28 C at various times during viral replication. The pattern of incorporation of the infected cultures at 28 C indicates that the rate of viral RNA synthesis can be greatly reduced if incubation at 28 C is initiated at about 1.5 hr after infection.

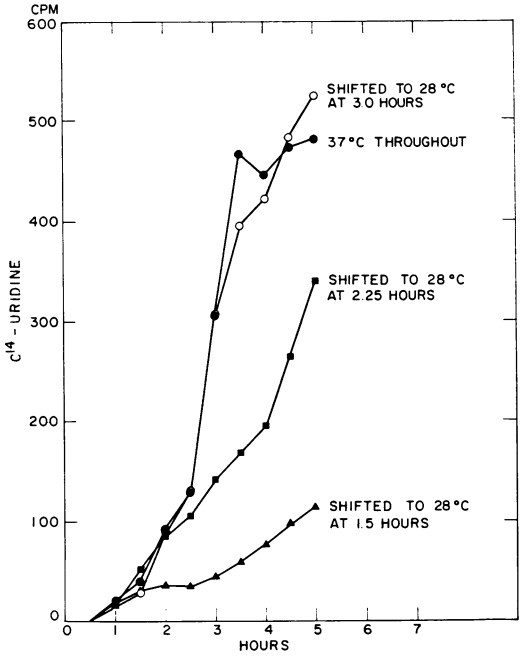

FIG. 5. *Mengovirus RNA synthesis in HeLa cells at 37 and 28 C. Each of four replicate cultures was infected at 20 PFU of mengovirus per cell at time zero at 37 C in the presence of 5 μg of actinomycin per ml. At 0.5 hr, 0.1 μc of ^{14}C-uridine (specific activity, 25.5 μc/μmole) per ml was added to each culture. At the times indicated, cultures were shifted to 28 C. RNA synthesis was assayed throughout the incubation.*

Summarizing the last two experimental findings, it may be concluded that incubation of poliovirus-infected cells at 28 C allows viral-induced inhibition of host cell protein synthesis to occur. At the same temperature, mengovirus RNA synthesis is greatly reduced. These facts were utilized to determine whether mengovirus development in HeLa cells preinfected with nonreplicating poliovirus could be rendered sensitive to the protein synthesis inhibitory action of the poliovirus.

In such an experiment (Fig. 6), conditions of the cultures were the same as in Fig. 1 except that some cultures were incubated at 28 C for 1 or 2 hr, starting at 1.5 hr after mengovirus infection. Curves for cultures maintained at 37 C throughout the experiment (Fig. 6A) are similar to those of Fig. 1. Figures 6B and 6C were obtained for cultures maintained at 28 C for periods of 1 and 2 hr, respectively, during the mengovirus replication cycle. Although there was a lag in the rapid increase in mengovirus RNA synthesis corresponding to the lengths of the 28 C incubation periods, there was no difference in the lag observed in preinfected and nonpreinfected cultures. Thus, mengovirus protein synthesis remains unaffected when exposed to the poliovirus-produced inhibitory factor, even when mengovirus RNA synthesis is greatly reduced.

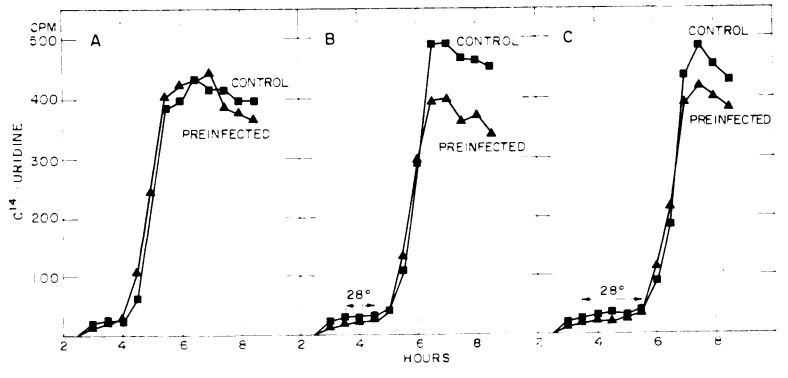

FIG. 6. *Mengovirus RNA synthesis in preinfected cells after 0, 1, and 2 hr of incubation at 28 C. HeLa cells were preinfected at time zero and superinfected at 2 hr as described. ^{14}C-uridine (0.1 μc/ml), with a specific activity of 25.5 μc/μmole, was added to each culture at 2.5 hr. Different pairs of preinfected and nonpreinfected cells were incubated at 28 C for the intervals indicated; RNA synthesis was assayed as in Fig. 1.*

DISCUSSION

The results of the studies reported here indicate that the replication of mengovirus proceeds normally in HeLa cells preinfected with nonreplicating poliovirus. Although it is demonstrated that infection with poliovirus sharply inhibits protein synthesis of the host cell, under these same conditions, no effect on mengovirus development can be detected. The experiments indicate that the lack of inhibition of mengovirus replication by nonreplicating poliovirus is real and not the result of the superinfecting virus outpacing the inhibitory factor.

Experimental conditions were employed to render mengovirus replication sensitive to the possible inhibitory action of poliovirus. Mengovirus replication was interrupted by inhibition of protein synthesis with Acti-Dione and by reducing RNA synthesis with low temperatures. The inhibitory effects of poliovirus on host protein synthesis are operative under these conditions of incubation. The results of both experiments lead to the conclusion that the inhibitory factor of nonreplicating poliovirus which inhibits host cell protein synthesis (8) does not inhibit the function of the mengovirus protein-synthesizing apparatus.

It is assumed, in the interpretation of these experiments, that the inhibitory agent produced by poliovirus is present during mengovirus growth. It is conceivable that superinfection with mengovirus interferes with poliovirus-directed protein synthesis. This is probably not a serious consideration, since previous experiments have shown that the inhibitory agent produced by poliovirus is stable and continues to function for at least 1 hr after the inhibition of virus-directed protein synthesis (8). Even if poliovirus-specific protein synthesis were suppressed, the inhibitory agent produced in the 2 hr prior to mengovirus superinfection should remain active during later stages of infection.

It has been shown that poliovirus exerts its inhibiting effect on host cell protein synthesis by interrupting the attachment of ribosomes to host messenger RNA (8). Recent experiments indicate that a species of host RNA is clearly involved in the selective inhibition of host synthesis. However, these experiments strongly indicate that the species of RNA might not be messenger RNA (W. McCormick, *unpublished data*). Our findings might, therefore, be explained by the possibility that the poliovirus inhibitory factor is able to distinguish between specific RNA elements involved for host cell and heterologous viral protein synthesis. Choppin and Holmes (1) have also made a similar proposal to explain the apparent lack of inhibitory effect by poliovirus on SV5 RNA replication.

A plausible explanation of the findings reported here is that viral protein synthesis has a different initiation signal than that of host messenger. Thus, poliovirus apparently interrupts host cell-directed intiation of translation, whereas viral initiation appears immune.

ACKNOWLEDGMENTS

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